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Title: A diagnostic test kit for determining a predisposition for breast and ovarian cancer, materials and methods for such determination.

5 The present invention relates generally to the field of human genetics. In particular the invention relates to methods and means (diagnostic test kits) for studying the predisposition for certain types of cancers often having a hereditary component and more specifically to the detection of a specific type of germline mutations in genes involved or associated with certain types of hereditary cancers, in particular the (human) BRCA1 gene, which will predispose to breast and ovarian cancer. In addition, the invention reveals
10 a molecular genetic mechanism that may have mediated the genesis of these mutations, in particular the role of Alu repetitive DNA elements present in the intronic regions of BRCA1. The invention further relates to somatic mutations of this type in the BRCA1 gene in human breast and ovarian
15 cancer, and their use in the diagnosis and prognosis of human breast and ovarian cancer.

The invention also relates to the screening of this type of BRCA1 mutations in human genomic DNA, as part of clinical protocols for the diagnosis of inherited predisposition to
20 breast and ovarian cancer.

Background of the invention

Breast cancer is the most common malignancy among women in the Netherlands, with a cumulative risk by age 85 of one
25 in 11. The strongest epidemiological risk factor for the disease is a positive family history. Depending on the age of diagnosis and occurrence of bilateral disease in the index case, first degree relatives may have a relative risk of up to 10 for developing breast cancer. In
30 the US population, 6 to 19% of women with breast cancer have at least one affected relative at the time of diagnosis [1],

but not all of them are expected to be true genetic cases as the high incidence of breast cancer in the general population will inevitably cause some coincidental familial clustering. In an attempt to stratify the two classes, criteria to define truly inherited breast cancer have been proposed [2]. Such cases are characterized by early age of onset (premenopausal), excess of bilaterality, and clear paternal or maternal transmission with an autosomal dominant mode of inheritance. Approximately 5% of all cases comply with these criteria, while another 13% are classified as familial clustering[3]. Since early age of onset appears to be a hallmark of hereditary breast cancer, one may suspect that among these cases the genetic component is much higher. Indeed, up to 36% of cases diagnosed under the age of 30 are expected to be genetic [4]. No such data are available for the Dutch situation, and little or none of this has been confirmed at the molecular genetic level.

Linkage analysis of early-onset breast cancer families localized BRCA1 to the long arm of chromosome 17 [5]. Further analyses of additional families revealed that women inheriting a mutant allele of BRCA1 are also at increased risk for ovarian cancer [6,7]. Overall, approximately 45% of all families in which breast cancer is the predominant malignancy are due to BRCA1, as are over 80% of all families with both breast and ovarian cancer [6,8]. Female mutation carriers have been estimated to have an 87% risk to develop breast cancer before the age of 70, and 63% risk to develop ovarian cancer before that age [7]. However, significant evidence for ovarian cancer risk heterogeneity was obtained, indicating the existence of at least two classes of BRCA1 mutations; one conferring a high risk to both breast and ovarian cancer, and one conferring a high risk to breast cancer, but only a moderate risk to ovarian cancer, with the former comprising approximately 26% of all BRCA1 mutations [9]. The gene frequency of BRCA1 has been estimated to be 1 in 833 women [10]. This would imply that 1.7% of all breast.

cancer patients diagnosed between age 20 and 70 are carrier of such a mutation.

The gene structure of BRCA1 was found to consist of 22 coding exons spanning >80 kb of genomic DNA [11], and encoding a 7.8 kb transcript [12]. An unusually large exon 11 of 3.4 kb comprises 61% of the coding domain. Over 900 mutations in BRCA1 have been published to date and compiled into an electronically accessible database [13]. Several characteristics stand out [14]. First, they are nearly ubiquitously distributed over the gene. Second, >85% of the mutations in the database lead to premature termination of protein translation. These include basepair substitutions leading to a stop codon, small insertions and deletions (of 1 to 40 basepairs) leading to a frame-shift, or splice-site mutations leading to deletions of complete exons and frame shifts. That these changes presumably inactivate gene function is supported by the finding that the great majority of breast and ovarian tumours that develop in BRCA1 mutation carriers show loss of the wildtype allele [15]. The relevance in terms of cancer predisposition of the missense mutations remains a matter of debate. Some of them appear rare polymorphic variants, as they are also observed in control samples. Others seem to affect critical residues, such as the cysteines in the amino-terminal ring finger domain [12], which are conserved in the mouse Brcal sequence [16]. Third, a number of mutations have been found repeatedly, reducing the number of distinct mutations to about 150. Two of these, the 185delAG mutation and the 5382insC mutation, each represent approximately 11% of all mutations thus far reported [14]. Reconstruction of the haplotypes bearing some of the most common mutations has provided strong evidence that they have either a single or a few common ancestors and may have been present in the population already for several centuries [17-19]. Consequently, the incidence of specific mutations is strongly dependent on the population from which the breast cancer families were ascertained. Thus the

185delAG mutation was picked up mainly in families of Ashkenazi-Jewish origin [20].

The extent of the founder-effect was highlighted by the finding that approximately 1% of all Ashkenazi Jews (i.e. regardless of a positive breast cancer family history) are carrying this mutation [21,22], 8 times that of the incidence of all mutations together in the general population [10]. Specific mutations have also been recurrently detected in breast cancer families of Swedish, British, Italian, and Austrian origin [18,23-26].

Despite the vast number of BRCA1 gene changes detected to date, there remains a discrepancy between the proportion of BRCA1 mutations predicted by linkage studies [6,8], and the actual prevalence established by mutation analysis, among breast cancer families derived from a variety of ethnic backgrounds [27-31]. In general, this is explained in two ways: either a substantial number of mutations have been missed by the applied mutation screening methodology, or the genetic heterogeneity of hereditary breast cancer is significantly greater than hitherto expected.

Relatively little information of predictive value can be gleaned from the existing data. In one set of 35 kindreds with proven BRCA1 mutations from the United Kingdom, the ovarian cancer risk heterogeneity as predicted from linkage studies could be confirmed [25]. Mutations occurring before codon 1435 conferred a significantly higher ovarian cancer risk than those occurring after this point. While this is consistent with earlier predictions based on linkage analysis [9], the current mutation distribution is at odds with the predicted lower frequency of these alleles. In addition, the expressivity of BRCA1 displays considerable inter-family variability. For example, the 185delAG mutation was detected in families with early-onset breast cancer and ovarian cancer, or late-onset breast cancer without ovarian cancer [32]. Clearly, other factors influence the expression of the

phenotype, and some of those might be genetic, others environmental. Of note, BRCA1 carriers who have a rare allele at the HRAS1 minisatellite locus were recently shown to be at a 2.8-fold increased risk for ovarian cancer relative to those carriers who had common alleles at HRAS1 [33]. However, a firm establishment of the full spectrum of BRCA1 gene changes in the population is pivotal for a more formal analysis of this matter.

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An intriguing feature of BRCA1, and unexpected in the light of Knudson's two-hit inactivation theorem for tumour suppressor genes, is that somatically acquired mutations are extremely rare in ovarian tumours [34-38] and have in fact not yet been detected in 135 breast tumours [39,40]. This might indicate that inactivation of BRCA1 is not selected for during tumorigenesis of the non-inherited form of breast cancer. BRCA1 expression might be critical only during certain stages of tissue development, e.g., during puberty when the breast undergoes its final differentiation into a potential milk-producing gland [39]. However, others have argued that the mechanism of inactivation might be different from that seen in inherited cases [41]. The present invention now reveals that the unusual high concentration of Alu-elements in the BRCA1 gene intronic regions [11] favors the induction of large genomic deletions and inversions in a situation of increased genomic instability although other mechanisms leading to these mutations may also play significant roles. The present invention thus provides a diagnostic test kit (and means and methods) for determining mutations, especially deletions of relatively large stretches of nucleotides in genes associated with hereditary types of cancer, in particular such mutations (deletions of relatively large stretches of nucleotides) in the BRCA1 gene. Such mutations are difficult, if not impossible, to detect by the currently PCR-based approach (if their occurrence or the site thereof is unknown) using genomic DNA as template, which has

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been most widely applied to establish the current mutation spectrum of BRCA1.

The present invention thus provides a diagnostic test kit for detecting the presence of or predisposition for e.g.

5 breast cancer, whereby a means is provided for detecting a deletion of a stretch of nucleotides from a BRCA 1 gene in a sample. Now that it is known that such mutations occur, it is within the skill of the art to arrive at means to determine the presence of these mutations, either the ones disclosed
10 herein or similar mutations. Such means may include hybridization of a probe flanking both sides of the deletion, or using two probes on either side of the deletion and amplifying the stretch in between, another way may be lack of hybridization, when using a probe hybridizing to a deleted
15 part, etc. Yet another way may be lack of amplification between one or more sets of primers targeted at or near a deleted region. This already implicates that typically multiplex PCR approaches are very suitable. Also exon-connection PCR is a very suitable approach for use in the
20 present invention. The techniques mentioned above are well known in the art and need no further explanation. Since mutations as disclosed herein may occur in one allele only, quantitative methods are often preferable. It is of course clear that the diagnostic test kit should provide all other
25 necessary means for determining the presence or absence of the mutations, such as buffers, detection means (possibly labels or markers), etc.

A convenient diagnostic test kit according to the invention apart from amplification methods such as PCR, NASBA and the
30 like is a diagnostic test kit whereby the means comprise the necessary elements for southern blotting. The deletions to be detected are typically relatively large stretches of nucleotides, particularly of a size which when subjected to PCR or similar amplification techniques would not be
35 amplified under normal reaction conditions because of their length. Typically the deletion comprises one or more exons of

the BRCA1 gene or a frameshift and/or a termination codon. An exemplified deletion that is a good marker for the predisposition for cancer is the deletion which comprises at least a major part of exon 22.

5 Another exemplified deletion that is a good marker for the predisposition for cancer is the deletion which comprises at least a major part of nucleotides 1396-1662.

Another exemplified deletion that is a good marker for the predisposition for cancer is the deletion which comprises at
10 least a major part of exons 13-16.

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15 predisposition for cancer is the deletion which comprises a stretch of nucleotides between two ALU-elements. This kind of deletion ties in very nicely with a suggested mechanism of the origin of these mutations and the same may also be found in other genes involved in cancer and having many of these
20 elements.

Thus the invention further provides a probe for use in a diagnostic test kit according to invention comprising a nucleic acid sequence which is a fusion of two (complementary sequences of) ALU elements, in particular of the BRCA1 gene.

25 In general the invention thus provides a probe for use in a diagnostic test kit according to the invention, which is a fusion product of two sequences adjacent to the site of a deletion of a stretch of nucleotides.

Also provided is a method for determining the presence
30 in a sample of a nucleic acid derived from a BRCA1 gene having a deletion of a stretch of nucleotides, comprising contacting said sample with at least one probe which alone or together with other means is capable of distinguishing between BRCA1 genes having said deletion and BRCA1 genes not
35 having said deletion, allowing for possible hybridization

Specific embodiments of the invention will be explained in detail below.

Detailed description of the invention.

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We have found that the mutation spectrum of BRCA1 as resolved up to this point [13,42] has been biased by PCR-based mutation-screening methods such as SSCP, the protein truncation test (PTT), and direct sequencing, using genomic DNA as template. We describe as examples thereof two large genomic deletions, which are not detected by these approaches, and which together comprise 38% of all BRCA1 mutations found in a sample of 170 Dutch breast cancer families [43,44]. One deletion removes 510 basepairs (bp) including exon 22 (Figure 1) and was found 8 times. The other deletion removes 3835 bp including exon 13 (Figure 2) and was found 4 times.

The haplotypes of the 8 families with the exon 22 deletion were reconstructed by typing 3 intragenic markers (D17S855, D17S1322, D17S1323) and 2 flanking markers (THRA1 and D17S1327). These haplotypes were completely concordant for the intragenic markers in at least 7 families, and the haplotype conservation extended proximally to THRA1, and distally to D17S1327, in at least 5 families, to comprise a genetic region of approximately 2 cM. The haplotypes of the 4 families with the exon 13 deletion were reconstructed in a.

similar way. These haplotypes were completely concordant for the intragenic markers in at least 2 families, and the haplotype conservation extended proximally to THRA1, and distally to D17S1327, in all 4 families, to comprise a genetic region of approximately 2 cM.

Molecular characterization of the deletions revealed that the exon 22 deletion starts in intron 21 and ends within the most upstream copy of three head-to-tail arranged Alu-elements in intron 22. A 17-bp imperfect homology to the intron 22 Alu-element was found at the 5' deletion breakpoint (Figure 3). The 3' breakpoint is closely flanked on either side by two 25-bp sequences strongly homologous to the Alu core-sequence implied to stimulate recombination [45].

The exon 13 deletion starts in intron 12 in an Alu-element (112 bp from the 5' end) and ends in intron 13 in a region which shares very high homology to this element (Figure 4). Both the 5' and the 3' breakpoint are closely flanked on either side by sequences strongly homologous to the 26-bp Alu core-sequence implied to stimulate recombination [45].

The current invention facilitates the design of PCR-based strategies (now that the presence of this kind of mutations is known) to identify the heterozygous presence of the deletions in human genomic DNA. Oligonucleotide primers can be designed so to immediately flank the deletion breakpoints, and allow the specific amplification of a deletion-junction fragment as a diagnostic endpoint. Given the size of the deletions, the wildtype BRCA1 genomic sequence would remain refractory to PCR-amplification under most standard reaction conditions. PCR-based diagnosis is an essential requirement to scale up throughput in the screening for these mutations.

The current invention also pertains to the molecular mechanism which may have generated the genomic deletions in the BRCA1 gene, especially since this needs to be viewed in a broader sense in that the same kind of phenomenon may be

picked up in other genes or in the same gene, but not having anything to do with the inheriting kind of cancer.

The current invention thus also pertains to the role of BRCA1 mutations in non-inherited or sporadic breast cancer.

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Experimental part.

The exon 22 deletion was revealed by Southern blot analysis of genomic DNA digested with either HindIII or BglII. As probe we used p1424, which contains ~1-kb cDNA-derived segment from exons 14-24. A carrier of the exon 22 deletion shows aberrant bands of 9.3 kb in the HindIII digest and of 6.7 kb in the BglII digest.

The exon 13 deletion was revealed by Southern blot analysis of genomic DNA digested with either HindIII or BglII. As probes we used either p11 or p1424, which contain ~1-kb cDNA-derived segments from exon 11 and exons 14-24, respectively. A carrier of the exon 13 deletion shows an aberrant band of 6.4 kb in the HindIII pattern obtained with probe p1424 and of 14 kb in the BglII pattern obtained with probe p11.

To further characterize these deletions, we used intronic amplimers to obtain PCR-products from genomic DNA, specifically containing the deletion-junction fragment. Amplimers flanking exon 22 generated an aberrant genomic fragment of 1.4 kb in DNA samples carrying the exon 22 deletion, which turned out to contain a 510-bp deletion relative to the wildtype sequence (Figure 3). The deletion affecting exon 22 removes the bases 79505-80014 (510 bp) as listed in the genomic sequence of BRCA1 (Genbank accession nr. L78833). As a result, 74 basepairs, corresponding to exon 22, are missing in the processed mRNA-transcript (bases 79543-79616 in Genbank accession nr. L78833).

Amplimers flanking exon 13 generated an aberrant genomic fragment of 2.7 kb in DNA samples carrying the exon 13 deletion, which turned out to contain a 3835 bp deletion relative to the wildtype sequence (Figure 4). The deletion

affecting exon 13 removes the bases 44514-48348 (3835 basepairs) as listed in the genomic sequence of BRCA1 (Genbank accession nr. L78833). As a result, 172 basepairs, corresponding to exon 13, are missing in the processed mRNA-transcript (nucleotides 46156-46327 in Genbank accession nr. L78833).

We examined 142 breast cancer families in which thusfar no BRCA1 or BRCA2 mutation had been found (refs. 43,44 and our unpublished results) for the presence of the exon 13 and exon 22 deletions. They were found in 4 and 8 families, respectively. Together with previous mutation screening results, using PTT and direct sequencing [44], these deletions thus comprise 12/32 (38%) of all families in which a BRCA1 mutation has been detected to date. Three intragenic and 2 flanking markers were used to reconstruct the disease haplotype for each of the research families carrying either the 510-bp or 3.8-kb deletion. Strong conservation of allele-lengths was observed at the intragenic loci among the haplotypes carrying the same deletion, in agreement with their descent from a common ancestor.

The haplotype in the Dutch population that carries the 510-bp deletion around exon 22 is characterized by a 155-bp allele at the microsatellite marker D17S855 in intron 20, a 122-bp allele at microsatellite marker D17S1322 in intron 19, and a 151-bp allele at microsatellite marker D17S1323 in intron 12. The haplotype in the Dutch population that carries the 3835-bp deletion around exon 13 is characterized by a 151-bp allele at D17S855, a 122-bp allele at D17S1322, and a 151-bp allele at D17S1323 in intron 12. The primer sequences used to detect these alleles are: for D17S1322: Forward (F) 5' CTAGCCTGGGCAACAAACGA 3' and Reverse (R) 5' GCAGGAAGCAGGAATGGAAC 3' for D17S855: F 5' GGATGGCCTTT TAGAAAGTGG 3' and R 5' ACACAGACTTGTCTACTGC 3' for D17S1323: F 5' TAGGAGATGGATTATTGGTG 3' and R 5' AAGCAACTTTGCAAT GAGTG 3'. PCR conditions have been described elsewhere [44].

Detection of the mutations

Isolation of genomic DNA and total RNA from freshly taken blood samples, and preparation of first-strand cDNA by reverse transcription, has been described [43].

cDNA analysis to detect the exon 13 deletion.

Exons 12-24 were amplified from first-strand cDNA products obtained by reverse transcription using the following primers for the first PCR: F (seq ID NO: 7) and R 5' GTAGCCAGGACAGTAGAAGGACTG (seq ID NO: 8) 3'. The obtained PCR-products were used as template for a second PCR of exons 12-24 using nested primers (F 5' GAAGAAAGAGGAACGGGCTTGG (seq ID NO: 9) and R 5' GGCCACTTTGTAAGCTCATTC (seq ID NO: 10) 3'). PCR conditions were as described previously [43]. Five µl of the final PCR products are analysed on a 1% agarose gel.

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Genomic PCR of the 3835-bp deletion spanning exon 13. A PCR reaction of 50 µl contains 200 ng of genomic DNA, 10 pmol primers (F 5' TAGGAGATGGATTATTGGTG (seq ID NO: 13) and R 5' TAC GTGGGTTCAACTGAAGC (seq ID NO: 14) 3'), 0.75 Units Amplitaq Taq polymerase (Perkin-Elmer-Cetus), and 5 µl of 10x ITP/BSA buffer (500 mM KCl, 100 mM TRIS-HCl pH 8.4, 25 mM MgCl₂, 2 mg/ml BSA, 2 mM dNTPs). This mixture is heated at 94°C for 5 minutes, followed by 35 cycles of PCR (at 94°C for 45 seconds, at 52°C for 1 min. and at 72°C for

2.5 min on a Perkin-Elmer-Cetus DNA thermal Cycler). The PCR is concluded by an incubation at 72°C for 6 minutes. Five µl of the PCR products are analysed on a 1% agarose gel.

Genomic PCR of the 510-bp deletion spanning exon 22. A PCR reaction of 50 µl contains 300 ng of genomic DNA, 10 pmol primers (F 5' TCCCATTGAGAGGTCTTGCT 3' ^(seq ID NO: 14) and R 5' ACTGTGCTACTCAAGCACCA 3' ^(seq ID NO: 15)), 0.75 U Amplitaq Taq polymerase (Perkin-Elmer-Cetus), 5 µl Optiprime buffer #6 (Stratagene) and 0.1 mM dNTPs. Thermal cycles are as described for the deletion of 3.8 kb. Five µl of the PCR products are analysed on a 1.5% agarose gel.

Southern analysis.

Five µg of genomic DNA is digested with either the restriction endonuclease BglII or HindIII. Agarose gels (0.8%) are run at 30V for 16 hr in TAE buffer (40 mM Tris-HAC pH 8.3, 1 mM EDTA). Procedures for denaturing, and transferring the separated DNA to nylon membranes (Hybond N+, Amersham) have been described [46]. As probes we used PCR-products obtained from a clone containing the complete BRCA1-cDNA, and purified by using the QIAquick PCR Purification Kit from QIAGEN. Probe-11 (p11) derives entirely from exon 11 and was obtained with the primers F 5' GAAAAAAAAGTACAACCAAATGCC 3' ^(seq ID NO: 16) and R 5' AGCCCACTTCATTAGTACTGGAAC 3' ^(seq ID NO: 17) and probe-1424 (p1424) contains exons 14-24 and was obtained with the primers F 5' TACCCTATAAGCCAGAATCCAGAA 3' ^(seq ID NO: 18) and R 5' GGCCACTTTGTAAGC TCATTC 3' ^(seq ID NO: 19). Purified fragments were labelled using the Megaprime DNA labelling System from Amersham according to suppliers protocols. Hybridizations were carried out at 65°C in 125 mM Na₂HPO₄·2H₂O, 7% SDS, 10% PEG-6000, 1 mM EDTA. Final washing was in 45 mM NaCl, 4.5 mM Na-citrate pH 7.0, 0.1% SDS, at 65 ° C for 30 minutes.

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Brief description of the drawings

Figure 1. Schematic representation of the genomic deletion spanning exon 22 of BRCA1. The intronic regions are drawn to scale relative to one another, and the exonic region are drawn to scale relative to one another, but not to intronic regions. The positions of the restriction endonucleases HindIII and BglII, used in Southern blot analysis, are indicated. The arrows indicate the presence and orientation of an Alu-element.

Sequence of the deletion

Figure 2. Sequence of exon 22 (upper case) and its flanking intron-sequence (lower case). ^(seq ID NO: 20) The numbers refer to the genomic sequence of BRCA1 (Genbank accession nr. L78833). Starting and ending positions of the 510-bp deletion are indicated by hooked arrows and affect positions 79505-80014. The first 241 bp of an Alu-element are depicted in italics, and the boxed sequences are imperfect copies (1 and 5 mismatches, respectively) of a common 26-bp core sequence involved in recombinations leading to gene rearrangements in the LDLR gene [45]. A stretch of 17 bp at the 5' junction of the deletion is homologous to a 19-bp stretch 37 bp upstream of the 3' deletion-breakpoint (underlined with arrows).

Figure 3. Schematic representation of the genomic deletion spanning exon 13 of BRCA1. The intronic regions are drawn to scale relative to one another, and the exonic region are drawn to scale relative to one another, but not to intronic regions. The positions of the restriction endonucleases HindIII and BglII, used in Southern blot analysis, are indicated. The arrowheads indicate the presence and orientation of an Alu-element.

Figure 4. Aligned sequences of intronic regions flanking exon 13 ^(seq ID NOS 21-22) and of the deletion-junction fragment (Jnctn) ^(seq ID NO: 23). The upper sequence of each alignment corresponds to intron 12

(seq ID No. 21)

(seq ID No. 22)

sequences, the lower sequence intron 13 sequences. The numbers refer to the genomic sequence of BRCA1 (Genbank accession nr. L78833). The boxed sequence indicates the 10 bp where the recombination took place that led to the deletion of 3835 bp. The intron 12 sequence depicted here represents the first 180 bp of an Alu-element. The intron 12 region 44481-44551 shares an 85% identity with the intron 13 region 48316-48386. The underlined sequences are imperfect copies of a common 26-bp core sequence involved in recombinations leading to gene rearrangements in the LDLR gene [45].

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